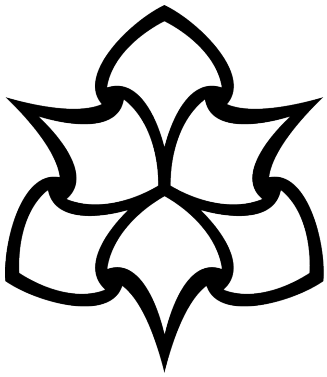

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ASSOCIATIONS OF SPERM TELOMERE LENGTH WITH SEMEN PARAMETERS, CLINICAL OUTCOMES AND LIFESTYLE FACTORS IN HUMAN NORMOZOOSPERMIC SAMPLES.

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Abstract

Background: Many studies have demonstrated that lifestyle factors can affect sperm quality and fertility. Sperm Telomere Length (STL) has been reported as potential biomarker or sperm quality. However, no studies have investigated the how lifestyle factors can effect STL and associated clinical outcomes.

Objectives: The purpose of this manuscript is to investigate any association between STL with lifestyle factors, semen parameters and clinical outcomes.

Materials and methods: Sperm Telomere Length was measured using real-time PCR in normozoospermic male partners (n = 66) of couples undergoing ART treatment. Each participant also completed a detailed questionnaire about general lifestyle. Linear regression univariate and

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ANCOVA analyses were performed to respectively determine correlations between STL and study parameters or identify statistically significant differences in STL while controlling for age, BMI and other factors.

Results: Using a linear regression model, STL is positively correlated with *in vitro* fertilisation success (n = 65, r = 0.37, p = 0.004) but not with embryo cleavage rates and post-implantation clinical outcomes including gestational age-adjusted birth weight. No associations were observed between STL and sperm count, concentration or progressive motility. We further found that STL did not associate age, BMI, health or lifestyle factors.

Discussion: In somatic cells - The rate of telomere shortening is influenced by a number of lifestyle factors such as: smoking, diet and occupation. However, little is known about how lifestyle factors affect STL and subsequently reproductive outcome.

Out data suggest that STL might have an important role mechanistically for fertilisation rate regardless of sperm parameters and lifestyle factors

Conclusion: The results of this study demonstrate that STL is associated with *in vitro* fertilization rates, but not with semen parameters nor lifestyle factors. Further investigations are warranted to identify the potential variation of STL overtime to clarify its significance as a potential biomarker in ART

Keywords

Sperm telomere length, lifestyle factors, fertilisation, semen parameters, male infertility

Introduction

Telomeres are complex ribonucleoprotein structures with repetitive DNA sequences (5'-TTAGG-3') that function as chromosomal stabilizing elements (Levy et al. 1992, O'Sullivan and Karlseder 2010). Repetitive cell divisions lead to telomere attrition (Allsopp et al. 1995). Once telomeres reach a critical short length, genome stability and cell division are affected which can result in an increase in DNA damage and cell apoptosis (Lazzerini-Denchi and Sfeir 2016). Furthermore, the rate of telomere shortening can be accelerated by the impact of lifestyle factors, environmental agents and increased activity of reactive oxygen species (ROS) (Song et al. 2010, Coluzzi et al. 2014, Ling et al. 2016, Cattan et al. 2008).

In male germ cell development, telomere length increases from spermatogonia to spermatozoa and is inversely correlated with the expression of telomerase activity (Ozturk 2015, Achi, Ravindranath and Dym 2000). In human sperm cells, sperm telomere length (STL) increases with age and positively correlates with male parental age at conception (Eisenberg, Hayes and Kuzawa 2012, Unryn, Cook and Riabowol 2005). In a young student population, Ferlin et al. first demonstrated a shorter STL in men having a low sperm count (threshold = 39 million), following (WHO 2010) compared to normozoospermic subjects and a significant and positive correlation between STL and sperm count (Ferlin et al. 2013). In another study from the same research group, a positive correlation between STL and sperm count in a normozoospermic selection of the population was demonstrated (Rocca et al. 2016). Additionally, men with idiopathic infertility or having varicocele have lower sperm counts and shorter STLs compared to fertile men who recently fathered a child (Thilagavathi et al. 2013a, Tahamtan et al. 2019). Furthermore, Lafuente et al. demonstrated that STL in infertile men was negatively correlated with sperm concentration and progressive motility (Lafuente et al. 2018). An increasing number of studies have found that STL may play a role in reproduction as a molecular marker of sperm quality.

Investigating the link between STL and fertility outcomes, Yang et al. showed STL was positively associated with the quality of embryos generated in clinical Assisted Reproductive Technologies (ART). Moreover, there was no significant association between STL and the fertilisation rate (Yang et al. 2015). Additionally, the reduction in STL was associated with an increased DNA fragmentation index (Tahamtan et al. 2019). DNA damaged sperm give rise to reduced embryo quality and development, but fertilisation is not impaired, and pregnancy outcomes are not strongly affected (Zini et al. 2005). In another study, the occurrence of an ongoing pregnancy after *in vitro* fertilization (IVF) treatment was null in a limited number of patients with abnormal relative STL (Cariati et al. 2016). Therefore, STL measurement is suggested to predict clinical outcomes (Boniewska-Bernacka, Panczyszyn and Cybulska 2018).

Several studies have demonstrated an impact of lifestyle factors on telomere lengths, mainly in leukocytes (for review, (Balan, Decottignies and Deldicque 2018). Lifestyle factors have also been stated to potentially affect sperm quality and fertility (Veron et al. 2018). However, no studies have investigated the association of STL and lifestyle with sperm quality and ART outcomes. The aim of this study was to investigate any association between human STL with normozoospermic sperm parameters and clinical ART outcomes, and its potential link to participants' lifestyles.

Materials and methods

Study subjects and sample preparation

Between 2013 and 2015, male partners of couples undergoing ART treatment at St Mary's Hospital Manchester were recruited for research with fully informed consent in writing with Local Ethics Committee Approval (Central Manchester REC ERP/91/078) and Human Fertility and Embryology Authority research licence R0026. 94 subjects' sample and data were randomly allocated for this study (including 3 which had to be discarded due to confounding anomalies or failure of oocytes culture).

Semen samples were produced by masturbation after 2 - 5 days of abstinence. Semen analysis was carried out according to World Health Organization (WHO 2010) guidelines. Briefly, following semen liquefaction after 30 - 60 minutes, semen volume was calculated by subtracting the weight of a pre-weighted container from the weight of the container with the semen. Sperm concentration was measured using a haemocytometer and total count was calculated by multiplying the sperm concentration by the volume of the whole ejaculate. Sperm motility was assessed by measuring the percentages of progressive, non-progressive and immotile sperm, according to the WHO guidelines,.

For this study, only subjects with a normozoospermic semen sample (total sperm count ≥ 39 x 10^6 / ejaculate, concentration ≥ 15 x 10^6 / ml, progressive motility ≥ 32 %, semen volume ≥ 1.5 ml, 66 participants) were included leading to a total study population of 66 male patients for STL analysis (Figure 1). After collecting the remaining of the sample from the hospital, the neat semen was centrifuged and washed to separate the sperm from the seminal plasma. Sample sperm concentration was measured (see above) and the sample was pelleted and frozen at -80°C until sperm DNA extraction.

Sperm DNA extraction

DNA was extracted from up to 25 x 10^6 sperm using the Qiagen Blood and Tissue Midi Kit (Qiagen, UK). Sperm pellets were incubated at 4°C overnight in the lysis buffer supplemented with RNase (10 mg / ml, Sigma, UK) and Proteinase K (1 mg / ml, Sigma, UK). To complete sperm lysis, a dithiothreitol solution (5 mM, Sigma, UK) was added and incubated first at 4°C for 1 hour on a rotator and second placed at 37°C for 1 hour. Following the manufacturer's instructions, the samples were added into gravity-flow columns. Eluted from the columns, DNA was isopropanol- and ethanol-precipitated and rapidly air dried to remove alcohol residual. DNA was quantified in TE buffer using a Synergene HT nucleic acid plate (Biotek, UK).

Sperm Telomere Length measurement

The STL of the DNA samples was analyzed using Real-time PCR, as previously described (Cawthon 2002), with some adjustments. Amplification of the telomeres were performed using the primers Tel O Forward (5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3') and Tel O Reverse (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3') and the two control genes were amplified using Col6a1 Forward (5'-TCACTCCTCTCCCTGTGGTTAT-3'), Col6a1 Reverse (5'-GGTTGACCTCGGTGGATGTG-3'), Col3a1 Forward (5'-ATGCACGTCTACATTAAGGAACTC-3') and Col3a1 Reverse (5'-TGCCCTCAGTGTCCAGTATG-3') using iTaq™ Universal SYBR® Green Supermix (BioRad). All of the samples were run on 96-well plates using a 7500 Real-Time PCR System (Applied Biosystems, USA). Amplification of the telomeric repeat region (T) was expressed relative to the amplification of the two control genes Col3a1 and Col6a1, single copy genes (S) located respectively on chromosome 2 and chromosome 21. The telomere to single copy (T/S) ratio is proportional to the average telomere length of the sample as the amplification is proportional to the number of primer-binding sites in the first cycle of the PCR reaction. Standard curves were used to ensure no great experimental variation between plates. Each sample's STL was calculated using a formula: $\Delta Ct_{sample} = ((Ct_T - Ct_{Col3a1}) + (CtT - Ct_{Col6a1})) / 2$. A significant positive correlation was observed between ΔCt values of Telomere-Col3a1 and Telomere-Col6a1 ($r = 0.491$, $p < 0.0001$, $n=66$). Relative STLs were obtained by normalizing the values against the mean of all STL measures. The coefficient of variation of samples' STL was 8.3%. One STL measure was determined to be an outlier (more than 2 standard deviation variable from STL mean) and excluded from the dataset (Figure 1).

ART procedures

Ovulation induction was achieved using conventional down-regulation involving pituitary desensitization. Recombinant Follicle Stimulating Hormone (FSH) was administrated by a step-

down protocol. When three or more follicles reached ≥17 mm diameter, beta human chorionic gonadotropin (β-hCG) hormone was administered and oocytes were recovered 36 hour later by ultrasound-guided retrieval. In *in vitro* fertilization (IVF) treatment, metaphase II (MII) oocytes were inseminated with ~1×10^5 prepared sperm/ml in IVF culture medium (G-IVF, Vitrolife) at 37˚C, 6% CO_2. In intracytoplasmic sperm injection (ICSI) treatment, a single motile sperm with apparent normal morphology was microinjected into each MII oocyte. Oocytes were examined 16-18 hours after insemination for the presence of two pronuclei, which is indicative of successful fertilization. Thus, the fertilized oocytes were *in vitro* cultured and their development was monitored. After 3 or 5 days, one or two good quality graded embryos were transferred into the uterus. After a fresh ART treatment, any spare embryos could be stored for future use, depending on their quality. Embryo implantation was assessed 12-14 days after fertilization by measuring the level of β-hCG hormone in the woman blood. Clinical pregnancy was confirmed 5-7 weeks after embryo transfer by the presence of gestational sac (fetal heart) detected by ultrasound scan. Biochemical pregnancy (early miscarriage) and late miscarriages were also recorded and were respectively identified as the absence of a fetal heart after a positive β-hCG and a pregnancy loss occurring after the detection of fetal heart. Births were reported including gestational age at delivery, birthweight and gestational age-adjusted birthweight.

Clinical ART outcomes analysis

Clinical ART data were extracted from the current integrated data management system (ACUBase) at St Mary's Hospital, Manchester. Each clinical ART outcome was analysed combining data from both IVF and ICSI treatment. Fertilization rates were calculated using the number of embryos at the pronuclei stage divided by the total number of either microinjected or inseminated oocytes. Embryo cleavage rates were calculated as the number of cleaved embryos 2-3 days after fertilization divided by the total number of fertilized oocytes. Ectopic pregnancy was counted as a successful pregnancy. To analyse the gestational age at birth and birth weights, these outcomes were referred to live birth events and only birthweights from singleton pregnancies were analysed due to the major biological characteristics of twin pregnancies. Gestational age-adjusted birth weights were calculated for using the Gestation-Related Optimal Weight (GROW) formula as described in (Castillo et al. 2019).

Lifestyle and Diet Questionnaire analysis

Participants completed a detailed questionnaire on general lifestyle exposures in the last 24 hours and over the past 3 months prior to ART treatment. The questionnaire was always

completed on the day eggs were recovered from the female partner, with the sperm samples produced on the same day and added to the eggs for fertilisation that day, and the remainder donated to research. The questionnaire composed of 23 questions and 1 food frequency table to determine the participants' parameters: biometrics (height and weight), ethnicity, education, general health (flu and work illnesses), lifestyle factors (physical activity, caffeine / alcohol consumption and smoking history) and dietary factors (patterns and food consumption frequencies). In relation to SLT, all lifestyle factors, except education, were analysed using a binary categorisation such as "yes / no" or applying a selected threshold. All factors containing continuous numerical data were also analysed to their respective STL values using direct correlation. In this cohort, 64 males out 65 were White Europeans. To examine biometric factors, thresholds for age and BMI were respectively applied at 35 years (starting age for male fertility decline, (Stone et al. 2013) and 25 kg / m^2 (to separate underweight / normal and overweight / obese). Weekly caffeine intake and alcohol consumption were calculated and reported respectively in mg of caffeine and alcohol units of alcohol following guidelines (Modi et al. 2010, DH 2008). Physical activity was analysed using both a binary score linked to the number of exercise hours and the Godin-Shepard Leisure Score Index (LSI)'s threshold defined at 24 (Amireault and Godin 2015). The unit had a strict policy to not treat smokers, hence no participants identified themselves as being a current smoker. Regarding the food frequency analysis (units per week), all single food items were grouped into food groups such as red meat, poultry and cruciferous vegetables. Food super-categories were created from food groups following similar biological classification (i.e. vegetables include cruciferous, leafy green and other vegetables; presented with a "1" exponent in Table 6). Two patients had minor missing information about consumption frequencies in the questionnaire and were excluded from the STL and food frequency analysis (Figure 1) which was carried out on 63 males of the cohort in Table 6.

Statistical analysis

Data are represented as mean ± the standard deviation (SD) or median with the interquartile range (IQR) using GraphPad Prism (La Jolla, CA) and IBM SPSS (Chicago, IL) software. Data normality was assessed using the Shapiro Wilk normality test. Depending on normality results, either parametric Welch's t-test or non-parametric Wilcoxon Mann Whitney tests were performed to determine differences between two study categories for biometrics, health and education / jobs data (expect for education level, Bonferroni's one-way ANOVA, Table 5). ANCOVA tests were performed to test differences while justifying for male age and BMI between study categories for ART outcomes (Table 4) and lifestyle / dietary factors (Table 5). Linear regression analyses were

performed using a general linear model univariate to determine correlations between STL and study parameters while adjusting for age, BMI and sporadically any other appropriate factors (sexual abstinence for sperm parameters in Table 3 and number of oocytes or embryos for ART outcomes in Table 4). P-values < 0.05 were considered statistically significant. P-values < 0.08 were reported as non-significant trends.

Results

General semen characteristics and clinical outcomes

Male biometrics and basic semen characteristics are shown in Table 1 and are reported as the mean +/- standard deviation (SD) or as the median (interquartile range, IQR) and minimal-maximal values. All normozoospermic semen parameters are based on the WHO lower reference values, with values greater than the lower reference values established by WHO 2010 guidelines (WHO 2010). ART clinical outcomes are presented in Table 2 showing the 65 couples achieving fertilisation using either IVF (n=48) or ICSI treatment (n=17), with 62 of them obtaining embryo cleavage. Two couples had their embryos frozen and transferred during a later cycle. Out of 60 fresh embryo transfers, 23 pregnancies (including 1 ectopic pregnancy and 3 early miscarriages prior 6-week scan) occurred resulting in 21 live births and 1 still birth. From the 62 couples with cleaved embryos, 33 had embryos frozen for future embryo transfer. Subsequently, 25 couples had their embryos thawed and 24 underwent a single or multiple frozen embryo transfers resulting in an additional 14 pregnancies and 9 live births. In total, 23 singletons and 4 sets of twins were born from 97 cumulative embryo transfers. Only one couple achieved two live birth events from fresh and frozen embryo transfers.

Sperm telomere length and normozoospermic semen parameters

No direct associations were observed between STL and semen parameters including total sperm count, concentration, semen volume, progressive motility, grade-A motility, percentage of immotile sperms and sexual abstinence in a normozoospermic population (Table 3).

Sperm telomere length and clinical ART outcomes

STL, *in vitro* fertilization procedures and clinical outcomes are presented in Table 4. When comparing STL in the ART procedures, no significant differences were observed between IVF and ICSI and as such for the further analyses of STL with clinical ART outcomes we used the combined data from both treatments. This revealed that STL positively correlated with the rate of fertilisation (Figure 2, $r = 0.32$, $p = 0.0097$; adjusted for BMI: $r = 0.36$, $p = 0.004$). In a limited number of cases where all embryos failed to develop, these embryos tended to have been

fertilised from lower STL sperm. Nevertheless, for the couples who had cleaved embryos, there were no significant associations between STL and embryo cleavage rate. In cumulating outcomes from fresh and frozen cycles, we observed a non-significant trend where STL was higher in couples that had successfully implanted embryos compared to couples with failed implantation ($p = 0.051$). However, STL was not found significantly different or associated with post-implantation events including clinical pregnancy, successful live birth event and gestational age-adjusted birth weight.

Sperm telomere length, Biometrics, Education and General health.

Table 5 shows the correlation between STL and biometric factors. No significant difference in STL was identified between threshold-defined groups for age or BMI. The STL of the normozoospermic men demonstrated no correlation with age ($r = -0.10$, $p = 0.4528$) and BMI ($r = 0.06$, $p = 0.6575$). Additionally, no associations were found with the degree of education or between those who had worked in the last 24 hours prior to ART treatment. Approximately 12% of participants had suffered from flu and fever but no difference in STL was found ($p = 0.8180$). Participants suffering from an illness caused or made worse by work tended to have a lower STL ($p = 0.0575$).

Sperm telomere length, Lifestyle and Dietary factors

When investigating common lifestyle factors such as smoking, drinking caffeine and exercise which are known to influence leucocyte telomere length (*for review see* (Thilagavathi, Venkatesh and Dada 2013b), we found no differences in STL for each lifestyle factor (Table 6) and no direct associations with STL (data not shown): consumption of caffeine or alcohol (respectively $r = -0.06$, $p = 0.6235$ and $r = -0.07$, $p = 0.6518$), exercising ($r = -0.09$, $p = 0.4894$; LSI: $r = 0.02$, $p = 0.1150$) and smoking (non-smokers vs ex-smokers: $p = 0.4117$) or being exposed to cigarettes smoke ($p = 0.9426$).

Dietary patterns including meat consumption in the last 24 hours were also investigated together with STL (Table 5). All male participants had a meat-inclusive diet. The difference in STL was not significantly linked to either dietary pattern (meat only vs meat + fish) and meat consumption. Overall food consumption frequencies of the last three months prior ART treatment were analysed for food categories such as meat, dairy, fruit, vegetables and more (Table 6). None of the food categories and single food items (data not shown) were significantly associated with STL. The consumption of processed meat tended to be inversely correlated to STL ($r = -0.24$, $p = 0.068$), however, the correlation coefficient remains low.

Discussion

Exploring the role of STL in semen quality, male fertility and reproductive clinical outcomes is a growing area of interest in reproductive medicine. In this study, we identified a positive correlation between the STL and the fertilisation rate in normozoospermic samples and with a very close trend towards higher STL in successful embryo implantation rates. However, we found no association between STL and sperm parameters. Furthermore, occupational health, diet and lifestyle factors were not correlated with STL. Altogether, our data suggest that STL might have an important role mechanistically for fertilisation rate regardless of sperm parameters and lifestyle factors.

Recent studies demonstrated that STL decreased in ICSI patients with previous low fertilisation rate (n = 10) (Darmishonnejad et al. 2018) and positively correlated with embryo quality (Yang et al. 2015), which is in agreement with data presented in the present study. In assisted conception practice, it is accepted that implantation and pregnancy outcomes are improved by the transfer of good-quality embryos compared to low-quality ones (Gardner et al. 2000, Zhao, Yu and Zhang 2018). However, in a recent study, Cariati and colleagues noted that no ongoing pregnancies were observed from patients having an atypical STL following IVF (Cariati et al. 2016). In another study, it was demonstrated that sperm with shorter STL was associated with lower natural pregnancy rates compared to couples who did achieve pregnancy naturally with longer STL (Lafuente et al. 2018). Herein, a trend was observed in which sperm samples with higher STLs tend to have an increased embryo implantation rate. However, in the present study, we observed live birth rates occurred with some of the lowest and highest STL values in our cohort. However, there was no STL association with birth weight or gestational age. Therefore, it could be hypothesized that a higher STL may be associated with increased fertilization rates and subsequent increased embryo development / quality, leading to improved implantation rates and pregnancy outcomes. Decreased STL was repeatedly observed in patients with oligozoospermia compared to normozoospermic samples, indicating that shorter STL may be associated with impaired spermatogenesis through segregation errors during meiosis (Ferlin et al. 2013, Thilagavathi et al. 2013a, Lafuente et al. 2018, Cariati et al. 2016).

Studies investigating the association between STL and sperm quality have shown a positive correlation with total sperm count and STL (Ferlin et al. 2013, Cariati et al. 2016). In other studies using normozoospermic samples, STL was identified positively linked to sperm progressive motility / vitality and negatively to sperm DNA fragmentation (Rocca et al. 2016), but no association was detected with male age, sexual abstinence, total sperm count, concentration and morphology. However, in another study, there was no association noted between STL and sperm parameters (Thilagavathi et al. 2013a). In the present study, we found no correlation between

sperm motility, count or concentration with STL. The current assessment of sperm quality as using the WHO guidelines (WHO, 2010) do not take molecular andrology pathways (such as STL and sperm DNA integrity) into account (Krawetz, De Rooij and Hedger 2009). Therefore, the quality of sperm and its fertilisation capacity may be dependent on molecular integrity in addition to the conventional sperm parameters.

In humans, lifestyle and environmental factors are known to influence telomere length in leucocytes (Thilagavathi et al. 2013b, Starkweather et al. 2014, Shammas 2011). This telomere attrition is associated with smoking cigarettes, low physical activity and stress exposure (Astuti et al. 2017, Nomikos et al. 2018, Jiang et al. 2019), though a recent large-scale study with repeated measures did not support any effect of alcohol consumption on leukocyte telomer length (Weischer, Bojesen and Nordestgaard 2014). Importantly, however, these parameters have never been investigated in relation to STL. The results presented here suggest that neither occupational or lifestyle factors associate with STL.

Similar to the other lifestyle factors, dietary patterns and food frequencies have been associated negatively and positively to leukocyte telomer lengths. Shorter telomeres have been associated with the level of consumed total fat and saturated fatty acid (Mazidi, Banach and Kengne 2018) wheas, consumption of seeds, nuts, legumes and coffee correlated with increase telomerses. Further recent studies support that a diet with more plant-based food items and reduced processed meat might be beneficial for increased telomeres and longer life, according to research on the Mediterranean diet (Freitas-Simoes, Ros and Sala-Vila 2016, Freitas-Simoes et al. 2018). Interestignly, the effect of diet interventions has been shown to have only minor, to no effect on telomeres (Perez et al. 2017, Meinila et al. 2019). In our cohort, STL was not associated with any food categories (such as meats, vegetables, fruits, etc.) or specific food item consumption. Processed meat consumption had a non-significant, but mild negative association correlation with STL ($p = 0.068$). Therefore, it seems that dietary factors do not impair telomere lengths in sperm to the degree in leukocytes.

Our data concur with other reports demonstrating no significant link between BMI and STL (Yang et al. 2015). This may be due to in part by the complex lifestyle and biological confounding factors (Yang et al. 2016). In patients with high BMI (>28 kg/m^2) compared to normal BMI, increased Reactive Oxygen Species (ROS) activity in semen and sperm DNA fragmentation were found; these are factors linked to poorer sperm quality that may account for poorer IVF treatment outcomes (Tunc, Bakos and Tremellen 2011, Ma et al. 2019). STL attrition is linked to ROS exposure in relation to environmental and lifestyle factors such as alcohol consumption and smoking (Vecoli et al. 2017, Saleh et al. 2002, Silva et al. 2017). However, Mishra and colleagues

demonstrated that a mild increase in oxidative stress is beneficial for STL maintenance (Mishra et al. 2016).

Age is a well-investigated parameter for telomere lengths and advanced age is associated with a decrease in leukocytes (Muezzinler, Zaineddin and Brenner 2013) and increase in sperm (Eisenberg 2011). The ageing protection mechanism of Telomere length in leukocytes is well-defined in the literature whereas the mechanism of STL elongation remains currently unclear. This elongation phenomenon is inherited from the father to offspring independently to the offspring sex (De Meyer et al. 2007) and seems to be conserved across species (Pauliny et al. 2018, Bauch et al. 2019). Interestingly, this paternal inheritance is also evident in leukocytes (Nordfjall et al. 2005). Older parental age at conception has been shown to have a negative effect on both offspring viability in mice (Garcia-Palomares et al. 2009) and the overall fitness of offspring in humans (Simard, Laprise and Girard 2019, Khandwala et al. 2018). As a confounding factor, ageing is linked to fertility issues, such as the decline in male hormones and sperm quality (Almeida et al. 2017). Therefore, an age threshold is required to investigate the effect of age on STL (such as <30 years vs >50 years, (Kimura et al. 2008, Stone et al. 2013). Herein, the age threshold has matched the age considered the start of male fertility decline (35 years), (Stone et al. 2013). In the present study, age did not show any significant correlation with STL. However, the age group of the men in this study (25 to 45 years) is within to the “middle adulthood” group (25 to 44 years (UNSD 1982). Nevertheless, our study population with a small age and BMI range highly represents current male patients, as men with outlying ages and BMI, which can be found in other studies, are simply not treated.

This study sought to investigate any association with STL, lifestyle factors, sperm parameters and clinical outcome. However, we recognise some limitations of our study. We selected normozoospermic samples, which limited the number of samples available. Moreover, as the samples were only provided at a single time point – we were unable to control for variations between ejaculates, or test possible causal interactions to changes in lifestyle factors, STL and sperm parameters. In addition, as sperm DNA extraction was performed in pelleted sperm sampled, we appreciate possible contamination of leucocyte and round cells (RC). However, as these samples were used for clinical treatment there was minimal contamination noted. Furthermore, a recent study reported that specific markers indicate that seminal RCs are mostly immature post-meiotic germ cells (Palermo et al, 2016). Therefore, given the low number of possible leucocyte or RC relative to sperm cell numbers in our study samples, we are confident that the STL results are representative of the sperm.

This study demonstrates a positive association between STL and the fertilisation rate in a normozoospermic population. However, we did not find any association between the sperm

parameters and the individuals biometrics and lifestyle factors. The data presented in this present study sets the scene for larger longitudinal studies to explore the dynamics of STL in humans in relation to lifestyle influences and to clarify its significance as a potential biomarker in ART, and the inheritance effect of a longer STL on ART offspring's fertility.

Author's roles

M.C., C.M., D.R.B. and A.C.P. designed the study. C.N., B.A., G.P. and G.H. acquired the samples and clinical outcomes. C.N., G. P. and J.S. carried out the experimental work. S.C.B carried out the analysis. S.C.B and M.C. wrote the paper, which was edited by C.M., D.R.B. and A.C.P.

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Conflict of interest

None declared

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Flow chart showing exclusion criteria for s. ART: Assisted Reproductive Technologies,

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Table 1

Biometrics (n=65)		(min – max)
Age (years)[a]	35.53 ± 4.48	25.0 – 45.0
Body Mass Index (kg/m2)[a]	25.08 ± 3.16	17.5 – 33.3
Semen Parameters (n=65)		
Total sperm count ($x10^6$) (≥$39x10^6$*)[b]	191.2 (127 – 329.6)	46.8 – 600.0
Sperm concentration ($x10^6$/ml) (≥$15x10^6$/ml*)[b]	63 (39 – 94.5)	15.0 – 362.0
Semen volume (ml) (≥1.5 ml*)b	3.3 (2.2 – 4)	1.5 – 7.2
Progressive motility (%) (≥32%*)[a]	55.31 ± 10.95	34.0 – 83.0
Grade-A motility (%)[a]	35.59 ± 13.52	9.0 – 80.0
Immotile sperm (%)[a]	35.11 ± 9.48	13.0 – 63.0
Sexual abstinence (day)[a]	3.43 ± 0.75	2.0 – 5.0

Continuous data are presented as mean ± SD (a) or median (IQR) (b) with minimum and maximum values (min-max). The World Health Organisation lower references for human semen characteristics are notified with a star (WHO. 2010). n: number of participants.

Table 2

Clinical Outcomes	Full sample	Fresh ET	Frozen ET
Number of couples	65	65 (100%)	25 (38.5%)
Fertilization types	65 (100%)	-	-
IVF	48 (73.8%)	-	-
ICSI	17 (26.2%)		
Fertilization Rate (%)[a]	69.2 ± 20.4, n=65		
Embryo Cleavage	62 (95.4%)	-	-
Embryo Cleavage Rate (%)[b]	100 (62.5-100), n=62	-	-
Number of ET	97	60 (61.9%)	37 (38.1%)
Number of pregnancies	37 (38.1%)	23 (38.3%)	14 (37.8%)
Singleton pregnancy	23 (62.2%)	16 (69.6%)	7 (50%)
Twin pregnancy	4 (10.8%)	3 (13%)	1 (7.2%)
Early miscarriage	6 (16.2%)	3 (13%)	3 (21.4%)
Late miscarriage	3 (8.1%)	0 (0%)	3 (21.4%)
Ectopic	1 (2.7%)	1 (4.4%)	0 (0%)
Births	31	22 (71%)	9 (29%)
Singleton live births	23 (74.2%)	16 (72.3%)	7 (77.8%)
Twin live births	7 (22.6%)	5 (22.7%)	2 (22.2%)
Stillbirth	1 (3.2%)	1 (5%)	0 (0%)
Gestational age (weeks)[bc]	38 (37 – 39), n=23	38 (37 – 39), n=16	39 (38 – 40), n=7
Birthweight (kg)[bc]	3.345 (2.860 – 3.765), n=23	3.331 (2.569 – 3.556), n=16	3.765 (3.118 – 4.451), n=7
GA - adjusted[bc]	3.695 (3.337 – 4.266), n=23	3.596 (3.360 – 4.170), n=16	3.695 (3.337 – 4.622), n=7

Continuous variables are presented as mean ± SD (a) or median (IQR) (b). Categorical variables are presented as row total (percentage). Outcomes referring to live birth singleton events are notified by (c). n: number of events. IVF: *in vitro* Fertilization. ICSI: IntraCytoplasmic Sperm Injection. ET: Embryo Transfer.

Table 3

Semen parameters (n=65)	GLM Univariate r (p-values)
Total sperm count (x10^6)	0.01 (0.970)
Sperm concentration (x10^6/ml)	0.07 (0.579)
Semen volume (ml)	-0.12 (0.371)
Progressive motility (%)	-0.07 (0.622)
Grade-A motility (%)	-0.14 (0.291)
Immotile sperm (%)	-0.05 (0.680)

A general linear model (GLM) univariate was used to identify correlations between sperm telomere length and sperm parameters while adjusting for age, body mass index and sexual abstinence duration. n: number of semen samples; r: correlation coefficient.

Clinical Outcomes (n=65)

A.		STL mean ± SD, n	ANCOVA p-values	**B.**	r (p-values)
Procedures	IVF	1.013 ± 0.087, n=48	0.306	-	-
	ICSI	0.991 ± 0.074, n=17			
Fertilisation	Yes	1.007 ± 0.084, n=65	-	Fertilisation (%)	**0.32 (<0.010)**
	No	-		*(adjusted for male age, oocyte number and male BMI)*	**0.37 (0.004)**
Embryo cleavage	Yes	1.010 ± 0.084, n=62	0.079	Embryo cleavage (%)	0.00 (0.981)
	No	0.956 ± 0.029, n=3		*(adjusted for male age, embryo number and male BMI)*	0.07 (0.611)
Cumulative Pregnancy				Gestational age (weeks)	0.14 (0.514)
• Embryo Implantation	Yes	1.030 ± 0.012, n=32	0.051		
	No	0.988 ± 0.017, n=30			
• Clinical Pregnancy	Yes	1.026 ± 0.013, n=29	0.188		
	No	0.995 ± 0.016, n=33			
• Birth	Yes	1.029 ± 0.013, n=27	0.119		
	No	0.995 ± 0.016, n=35			
Birth weight (singleton)	< 2.5kg	1.022 ± 0.023, n=5	0.586	GA-Adjusted Birth weight (singleton, kg)	0.32 (0.137)
	≥ 2.5kg	1.038 ± 0.016, n=18			

Table 4

A: ANCOVA tests were performed to test differences in STL between groups while controlling for the number of embryos used, male age and BMI. **B:** Direct correlations between STL and continuous values of ART outcomes were assessed using Pearson's correlation tests. General linear model univariate analyses for fertilisation and embryo development rates were performed to identify associations with STL and adjust for male age, male body mass index and number of oocytes or embryos. STL: Sperm Telomere Length; BMI: Body Mass Index; GA: Gestational age; n: number of events; r: correlation coefficient.

Table 5

A. Biometrics (n=65)		STL, n	p-values
Age[a] (years)	< 35	1.016 ± 0.0795, n=26	0.4919
	≥ 35	1.001 ± 0.0868, n=39	
BMI[a] (kg/m^2)	< 25	1.007 ± 0.0927, n=35	0.9384
	≥ 25	1.008 ± 0.0732, n=30	
B. Health n=65)			
Fever / Flu[a]	Yes	1.013 ± 0.09121, n=7	0.8180
	No	1.006 ± 0.08234, n=58	
Illness (job)[a]	Yes	0.967 ± 0.06435, n=9	0.0575
	No	1.022 ± 0.08140, n=56	
Stress (job)[b]	Yes	0.9368 ± 0.07545, n=4	0.0820
	No	1.0170 ± 0.08155, n=61	
C. Education & Job (n=65)			
Education level[a]	Secondary school	0.966 ± 0.09748, n=9	0.1094
	GCSE or equivalent	1.040 ± 0.07605, n=20	
	A-Level	0.976 ± 0.07377, n=14	
	Undergraduate Level	1.011 ± 0.08701, n=13	
	Postgraduate Level	1.019 ± 0.07738, n=9	
At work (last 24 hours)[b]	Yes	1.006 (0.9409 – 1.079), n=47	0.8820
	No	1.017 (0.9384 – 1.051), n=16	

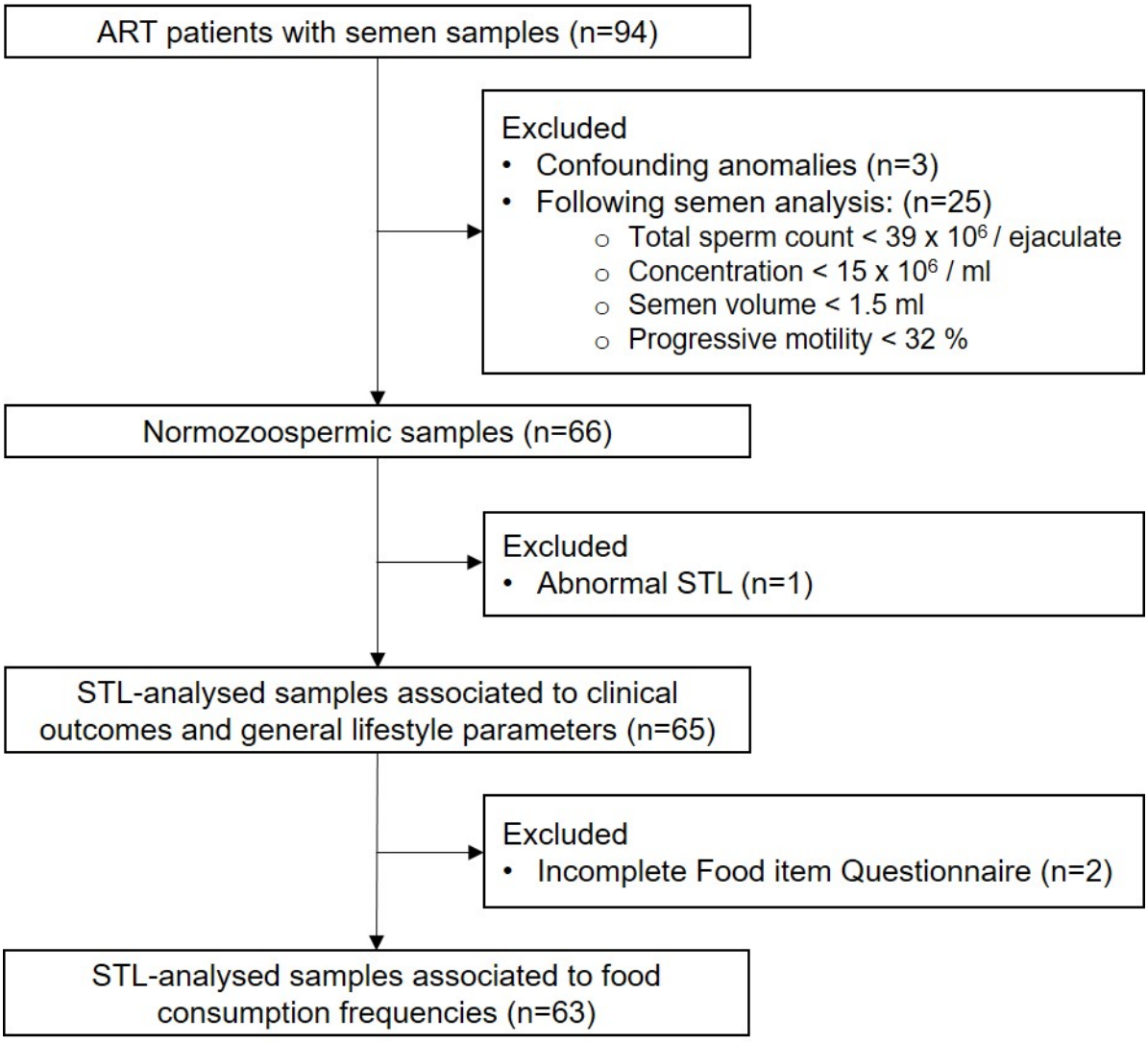
D. Lifestyle and Dietary factors (n=65)		STL, n	ANCOVA p-values
Caffeine intake[a]	Yes	1.007 ± 0.08411, n=64	-
	No	1.049, n=1	
Alcohol consumption[a]	Yes	1.002 ± 0.07566, n=44	0.611
	No	1.018 ± 0.09952, n=21	
Exercicing[a]	Yes	1.004 ± 0.08545, n=55	0.424
	No	1.025 ± 0.07428, n=10	
Leisure Score Index[a]	Inactive (< 24)	1.004 ± 0.08525, n=31	0.918
	Active (≥ 24)	1.010 ± 0.08307, n=34	
Smoking[a]	Ex-smoker	1.016 ± 0.07442, n=31	0.335
	Non-smoker	0.999 ± 0.09155, n=34	
Dietary patterns[a]	Meat	1.010 ± 0.09249, n=15	0.749
	Meat + Fish	1.006 ± 0.08177, n=50	
Meat consumption (last 24 hours)[a]	Yes	1.006 ± 0.08577, n=60	0.285
	No	1.018 ± 0.05677, n=5	

Continuous data are presented as mean ± SD (a) or median (IQR) (b). **A, B, C:** Differences in STL between groups were statistically analysed using either t-test with Welch's correction or Mann-Whitney test (expect for education level, Bonferroni One-Way ANOVA). **D:** ANCOVA tests were performed to test differences in STL between groups while controlling for male age and BMI. STL: Sperm Telomere Length; n: number of participants.

Table 6

Dietary Categories (n=63)	GLM Univariate r (p-values)
Meat[1]	-0.15 (0.263)
• Poultry	0.12 (0.337)
• Red meat	-0.07 (0.574)
• Processed meat	-0.24 (0.068)
• Fish	0.17 (0.202)
Dairies[1]	-0.08 (0.543)
• High-Fat dairy products	-0.08 (0.543)
• Low-Fat dairy products	-0.04 (0.790)
Fruits	-0.18 (0.175)
Vegetables[1]	-0.06 (0.628)
• Cruciferous vegetables	-0.03 (0.828)
• Leafy Green vegetables	0.08 (0.524)
• Other vegetables	-0.22 (0.085)
Nuts	0.19 (0.138)
Cereals and derived	-0.04 (0.742)
Cereals	-0.03 (0.804)
Confectionery	-0.12 (0.367)
Caffeinated no sugar beverages	-0.07 (0.570)
Caffeinated sugar beverages	-0.18 (0.159)
Vitamins	0.01 (0.956)

General linear model univariate analyses were performed to identify associations between STL and frequencies of food categories while adjusting for male age and male body mass index. Supercategories are notified by a “1” in exponent; n: number of participants; r: correlation coefficient.



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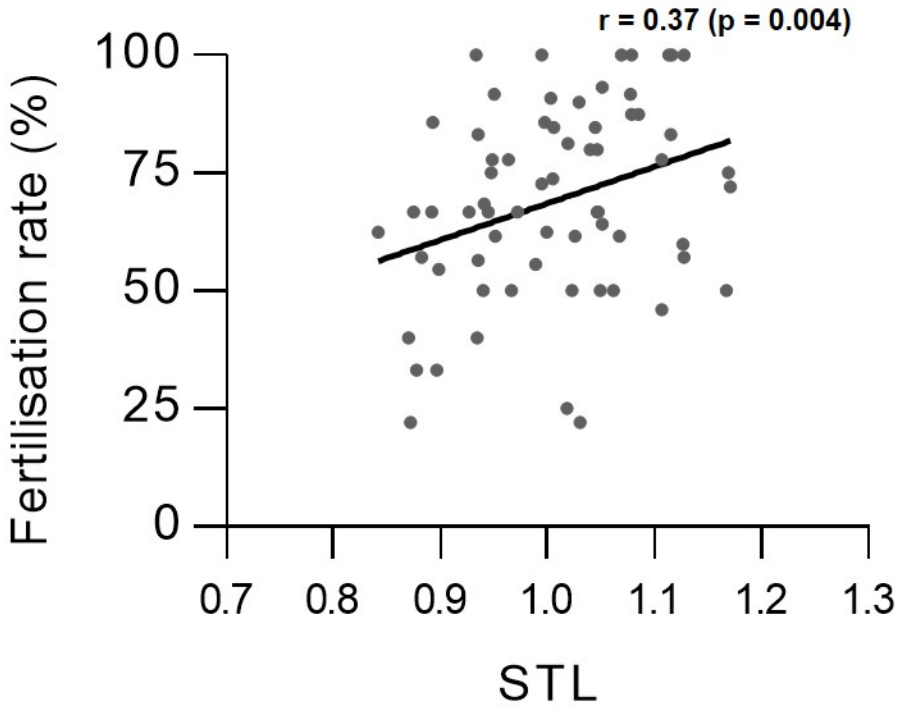

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